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Full Length Research Paper

The using of gibberellic acid hormone on cotton mature embryo resulted by crossing between wild and commercial species on artificial medium

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The wild species of cotton have important role in cotton breeding due to their favorable traits, which include pest and disease resistance, drought tolerance, fiber quality and male cytoplasmic sterility. Transferring these favorable genes from wild species to commercial cultivars of cotton by the traditional methods or classical plant breeding procedure will be very difficult and impossible. This is due to the following: Confronting disordering in flower structure, the problems involved in cotton flower pollens germination in stigma level and destruction of embryo and endosperm in different stages of evolution. The first sign of these problems is the falling of ovary. Therefore, the first and important barrier in crossing between diploid and tetraploid cotton species is the inability of hybrid seeds to produce. Solving of these problems can be the first step in hybridizations programs. The artificial control of fertilization by the gibberellic acid (GA_3) after crossing and hybrid embryo culture in the media will increase probability of hybrid plants production. In this study, three species named Sahel (tetraploid), Hashemabad (diploid) and Kashmar (diploid) were used. The parental species were planted on six plots and crosses were made between them. It must be noticed that we used Sahel species as paternal and the two diploid species as maternal species. As the study continues, two investigations were separately done on hybrids. Firstly, it consisted of using different amount of Gibberellic acid after pollination for the maintenance of bolls and secondly, 45 days embryos of all the crosses combined were cultured on liquid and solid M.S media. The results show that when hormone was not used, the amount of bolls that fell was 100%, but when Gibberellic acid was applied at 100ppm concentration, there was considerable differentiation in maintenance of bolls. The Hashemabad cultivar created bolls more than Kashmar but the percentage of seeds inside the bolls was so lower than Kashmar cultivar. In comparison between parents used and their hybrids, the latter's response was better. Also among the different media culture, it is seen that the growth of mature embryo in the liquid media was better than solid.

Key word: Cotton, diploid species, tetraploid species, hybrid, media culture.

INTRODUCTION

The idea of classical plant breeding is the transmission of biotic and abiotic stress resistance genes from wild species to the domestic species, termed the development of genetic ability (kumar, 1998). The numbers of Asian diploid wild species of cotton plant have been reduced many years ago. They are cultivated in limited region of India, China and Pakistan at present. Instead of that cultivation of upland cotton cultivar (*Gossypium hirsutum*) has been developed so that it dominates all kinds of cotton in the olden world. Therefore, extensive information about important genetic traits and their heredity like: Disease and pest resistance, tolerance to the unfavorable environment conditions, salinity and drought stress resistance and others have been created million years ago. These potentials can be transmitted to the commercial allo-tetraploid species for their genetic ability

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development. Of course, some have been eliminated from gene banks and forgotten (Rauf et al., 2004).

Cotton regeneration by callus or leave protoplast at *in vitro* conditions is very difficult. This problem has created a barrier to cotton breeding development (Bushra Rashid, et al., 2004).

The breeders have tried to improve cotton plants by classic methods that are used in cereal plants. Diseases and pests resistance does not exist in cotton germplast at the present. Therefore, these subjects have more research items in biotechnology and genetic engineering fields for attainment of new genetic information and for creating new varieties with recombinant characteristic (Seyed et al., 2005). Today, the different tissue culture methods are used for different goals such as: inter-species hybrids, production of mutants and variant hybrids, rapid and bulk plant production and etc (Lale, 2005).

For a long time, more cotton breeders and evolutionary researchers have searched about differences in crosses between new cotton diploid species and that of the olden world; and also different crosses between diploid and tetraploid cotton plants (Stewart, 1981). The attainment of some hybrids is difficult because there are incompatible factors in some cotton varieties. Introduction of gene between olden world (AA) and new world (AADD) cotton plants can be done in the limited range of species (Chengzhi et al., 1992).

Therefore, the understanding of these barriers and discovering their solutions in hybridization programs between cotton species is necessary. These problems are (Gill and Bajaj, 1987):

1. Before fertilization
a. Limited factors that prevent the transferring of a species pollens on another species, stigma.
b. Limited factors that prevent the germination of pollen and growing its tube and fertilization action.

2. After fertilization
a. Limited factors that prevent the hybrid seed (zygote) growth and embryodevelopment.
b. Limited factors that prevent the growth of hybrid sapling and mature plant.

For solving the aforementioned problems in inter-species cross process, variant ways have been introduced. For example; ovule culture methods (Bajaj and Gill, 1985; Stewart and Hsu, 1977, 1978) or the using of the gibberellic acid hormone in the crossing time (Altman, 1988).

These methods could facilitate hybridization and crossing between organisms approximately (Stewart, 1977; Brown et al., 1976).

On this basis, the first limited factor in crossing between diploid and tetraploid cotton species is seed production problem in hybrids. Before finding other problems in other steps of fertile hybrid plants production, it is necessary to choose which of the problems that must first be solved because that is the first step in hybridization program (Harlan and dewet, 1971).

In relation to this embryonic problem in interspecific hybrids, it can be said that between zygotic cells, endosperm and maternal tissues (ovule) there is no genetic or chromosomal proportionality. This is because endosperm tissue is only the fundamental feeding, hormone excitants and physiological source for embryo (Gill and Bajaj, 1987).

Successful embryo development in the first step depends on the natural growth of cells in the endosperm, embryo growth proportionality and also on the interaction hormone effects between embryo, endosperm and maternal tissue. All these factors are influenced by gene-tic or amount of chromosomes in each combined genome in cells and tissues (Khaghani, 1985).

The other method for interspecific crosses between diploid species and the upland cotton is crosses between two diploid species that duplicated their chromosome and then crossed it to the upland cotton (Brar et al., 1984). So the diploid species that are crossed with upland cotton directly or by using modern technology method will create sterile first generation (F1). For the production of fertile hexaploid, the sterile F1 hybrids should be treated with colchicine (Kulkarni, 2002; Davidonis et al., 1983).

The saving method of embryo was done by isolation of 3 days embryo (immature embryo). But in this way, and being that embryo developing period is prolonged, the production of seedling will be done by callus culture. The early falling of fertilized flower is caused by the isolation of mature embryos in relation to cotton interspecific crosses which is impossible; and it does not give a clear response. Therefore, in this study we have studied the treatment of the petioles, tetraploid and diploid cotton plants by GA_3 (a kind of growth hormone) immediately after fertilization to prevent flower from falling and for embryos to have enough time to develop in the ovule.

MATERIALS AND METHODS

Cultivars, planting method and crossing

Two interspecific crosses were done between one tetraploid cultivar (as paternal) and two diploid cultivars (as maternal) at the Hashem Abad Research Station in 2008. The used cultivars are seen in Table 1.

The selected cultivars were planted in the last 5th month or in May simultaneously. Each cross included 12 replications that are divided into two sections: 6 replications treated with hormone and other 6 replications not treated. Every plot had two rows of 8 m with 80 cm distance between them. The distance between plants was 40 cm. All cultivation and preserving methods were the same for cotton at Hashem Abad Research Station.

It must be noticed that flowering and pollination periods started in the environmental conditions of research station in July and continued till August. For the artificial pollination, the maternal flowers neutralized before maturation and pollination. For this reason, anthers are picked from stigma and after pollination with paternal pollens, the anthers are preserved from other pollens. The crosses in this test were between diploid cultivars as maternal and

Table 1. Some characteristics of the used cultivars.

Ploid	Cultivar name	Species scientific name	Genomic symbol
Diploid	Local Hashem Abad	*G. herbaceum*	A1
	Local Kashmar	*G. herbaceum*	A1
Tetraploid	Sahel (Sapied)	*G. hirsutum*	(AD)1

the tetra Ploid cultivar as paternal.

In each plot, two rows were considered for laboratory tissue culture studies. One hundred and eighty (180) flowers in each cross were treated with gibberellic hormone in 100 ppm (part per million (ppm)) concentration for preventing flower from falling and another 180 flowers in each cross were not treated with gibberellic hormone. Then, these flowers hybridized and their produced bolls were transmitted to laboratory for mature embryo culturing in *in vitro*. Finally, at the end of culturing season, all investigations were done on the remaining bolls of 720 crossed flowers in complete repeats.

The using of hormone material, its application and methods on the flowers

At the growth period, different concentrations of Gibberellic acid were used in order to prevent falling of boll of cotton hybrids. Based on other research results, we used 100 ppm concentration of this hormone in comparison to 0 ppm (as control).

In providing the hormone solution with considered concentration, a base solution (stock) was made for all treatments, first. For this reason, 100 mg gibberellic acid powder was placed in a balloon zhozhe with 100 cc volume, and then few drops of ethanol solvent were added to it; and after being completely solvated, its volume increased to 100 cc by distilled water.

For making 100 cc gibberellic acid solution with 100 mg per liter concentration, the volume of 10 cc made stock to increase to 100 cc by distilled water.

Treatments were applied on two parts of hybrid flowers which include attachment of petiole to shoot (for preserving boll from falling in cotton) and on ovary or inside part of brackets (for improving growth and developing conditions in inner tissues, ovary such as ovules and embryo). It was done by instilling one drop by drip pan.

Fertilizing embryo culture *in vitro*

This part of the research was done in the Biotechnology and Tissue Culture Laboratory in Cotton Research Institute of Iran.

Providing M.S. medium for ovules and hybrid embryos culture

For culturing of fertilized ovules *in vitro*, we should first provide culture medium. It was M.S medium in two liquid and solid forms.

The pollination, the conveyance of hybrid cotton bolls to laboratory and the mature embryos culture

Cotton flowers fertilization during the flowering period was done from July. 180 fertilized flowers of each combination cross were appropriate at this stage. In each mature embryo culture periods, some hybrid bolls after pollinating for 45 days (when the diameter of bolls reached 2 cm), were picked from maternal and conveyed to laboratory.

In the laboratory, after deleting brackets and fibers around the bolls and seeds respectively, the other stages such as: Washing them with water, cleaning materials, surface sterilization and fertilized mature embryos culture were done under x ray laminar hood before sterilizing with 70% ethanol; so all equipment were autoclaved. Therefore, *in vitro* culture was applied in sterile conditions. In this procedure, we sterilized seed surface with 70% ethanol and 50% hypochlorite sodium, then these seeds were put on medium surface (in solid medium) or on filter paper bridge (in liquid medium). Finally every plate had only one embryo.

After culturing the mature embryos on medium plates, pollination and culturing dates were recorded on every plate and then put in incubator. In it, cultured samples were in the dark (Ammirato and steward, 1971) and seedlings were produced under 30 ± 2°C conditions.

Investigation method of embryos' development and growth in *in vitro* culture

After one week of embryos culture, all infected cultured plates and necrosed embryos plates were omitted. Also, cultured ovules and embryos on M.S. medium were put in the glass pots with content of humus soil that was sterilized in 160°C for 3 h and transmitted to germinator finally. All the media (liquid and solid) contained 30 g/L sucrose for ovules culturing.

In each 20 liquid and solid media applied for every cross, firstly, they were the best media, secondly we used 10 hybrid embryos with calculation of deleted plates. At the end, we investigated the growth interaction of these cultured embryos this way: the relationship of germinated embryos percentage to all cultured embryos.

RESULTS AND DISCUSSION

After pollination and hormone treatment, the bolls falling started in hybrids cottons after 1st or 2nd day. It continued under farming condition for two weeks. Of course, the bolls falling were more in control (non treatment with gibberellic acid). The comparison of cotton treated with hormone and the control showed us that application of some hormone dosage reduce the hybrid bolls falling ratio considerably (Figure 1).

The hybrid cotton bolls were distinguished as those that receive hormone treatments and those in control (non-hormone treatment) after maturation and opening of them. Bolls treated with hormone had low fibers, and were malformed and immature compared to those in control. We applied the gibberellic acid hormone solution by dropping it at the end of the petioles. As a result, bolls'

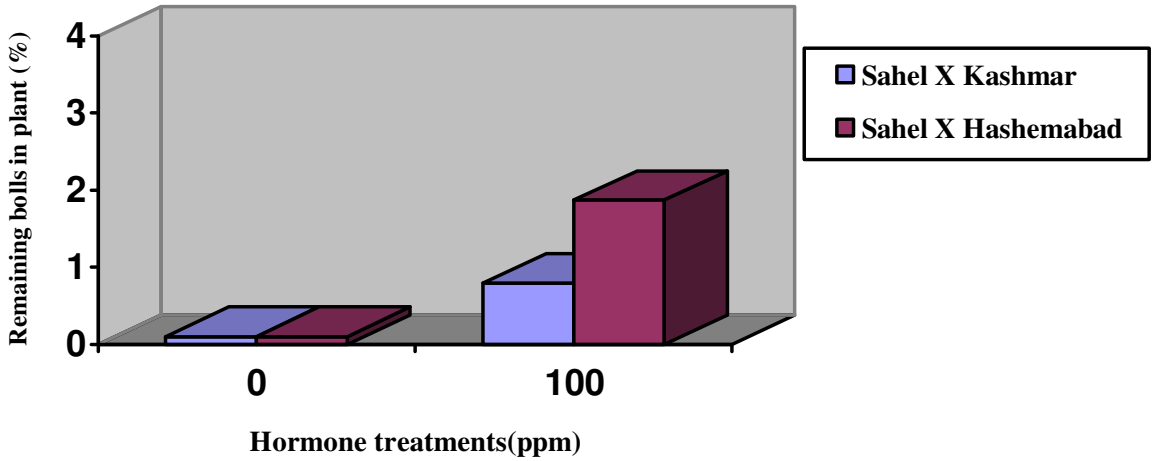

Figure 1. The interaction of hormone concentration and kind of hybrids on falling percentage in bolls.

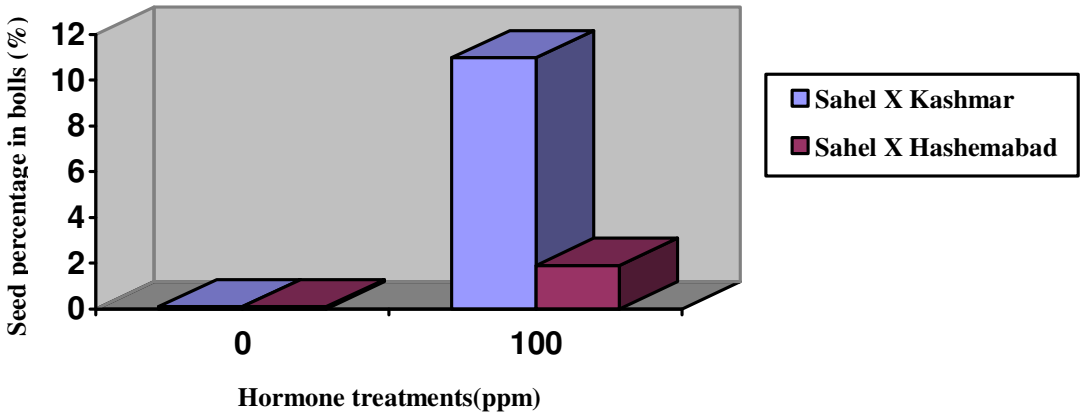

Figure 2. The interaction of hormone concentration and kind of hybrids on seed percentage in bolls.

falling was prevented by increasing tissue mass and making petioles steady at that point. Most of the remaining hybrid bolls were without seeds or with a small and wrinkled like seed.

These seeds included primary ovules which, in the first development stages, were allowed to develop and form some fibers on their outer surface; after which their growth stopped. Therefore, a few seeds were produced from the remaining hybrid bolls that were smaller than non-hybrid bolls. Each hybrid boll contained only 5-7 seeds (Figure 2).

Weaver and Pander (1957, 1958) and Panadir (1972) showed that when tetraploid species are used as the maternal in interspecific crosses, endosperms will be annihilated and embryos will be also destroyed, with bolls falling down finally. When diploid species are considered as the maternal in interspecific crosses, this abnormality is created in the endosperm and also in the embryo. For this reason, bolls falling will be higher in diploid maternal crosses in relation to maternal tetraploid crosses.

We must notice that in this design, hormone treatment was only done for the preservation of falling bolls; it did not have any effect on the number of boll seeds and it rarely caused enlargement of the size of the seeds.

Therefore, we can say that the results of this study on the numbers of bolls and hybrid seeds in interspecific crossing are better off than those of last researches. For example, Altman (1988) controlled the percent of bolls falling in some hybrids (crosses between diploid and tetraploid cottons) by using different effective hormone levels up to 100%. But these seeds were unable to germinate while obtained seeds in this study were able to germinate under farm conditions.

Gill and Bajaj (1987) applied a mixture of gibberellic acid hormone (belonging to the gibberellic group) and naphthalene acetic acid (belonging to artificial auxins), in the right and effective concentrations, several times on the fertilized flowers in a reciprocal crosses between diploid and tetraploid cotton genotypes. They increased the maintenance percent of hybrid cotton bolls up to 65.

Table 2. The average of germinated ovules in different used mediums and crosses.

Parent		Medium	
Maternal	**Paternal**	**Solid (%)**	**Liquid (%)**
Hashem Abad	Sahel	25	29
Kashmar	Sahel	33	41

But some could not grow in field conditions. Amin (1940) did interspecific crosses between diploid and tetraploid cottons without using any hormones in natural conditions. In this test, the result of the remaining hybrid bolls on the tetraploid and diploid cottons will be 0.7 and 0.1%, respectively. While we concluded that the maintenance percent of hybrid cotton bolls will increase by using hormones, for Hashem Abad and Kashmar genotypes, there were 3.03 and 1.41%, respectively.

The produced hybrid (that resulted from interspecific crosses between diploid and tetraploid cottons) seeds in comparison to parental ones' had smaller size, some were scarious and crusty; their fibers were fine and adhesive to seed shell also. Of course, we can say that the main reason is the non-proportionality between endosperm and chromosomes in ovule and embryo tissues. Following it, primary ovules will have little growth and after little fiber formation on the outer surface, their growth will be stopped.

According to Table 2, notwithstanding the parents, the liquid medium in comparison to solid medium produced more healthy embryos. Of course, the cross between Sahel and Kashmar varieties produced more germination in liquid culture medium. Based on the ultimate result of this experiment, the best method for producing hybrid cotton varieties from crossing between diploid and tetraploid parents in farm conditions by Gibberellic acid hormone is the using of 100 ppm GA concentration. This is because it can induce the prevention of bolls' falling, has lower cost and is not a difficult method in comparison to embryo culture.

The using of liquid media has been preferred to the nurse culture. The major reason of that is the lower taction of ovule and embryo to inhibitor materials that gradually accumulate in the medium and ultimately prevent growth and development of embryos.

Conclusion

Based on the result of this study, triploid plants produced by any means are proper resources for hexaploid plants production, with favorable diploid and high yield tetraploid species characteristics. In this study, triploid plants produced were larger than their parents. These plants are preserved as the germplasm in the greenhouse for further studies.

The two ways used in this research are apparatuses for removing the primary obstacle in interspecific crosses between diploid and tetraploid plants. We can say that these methods of producing hybrid seeds and mature embryos cultures are outstanding results of this project that can be used in other studies for increasing plant efficiency.

Also, we can increase seed production in interspecific crosses in future research. This s a small part of a research meant for the attainment of hexaploid cotton by treating triploid cotton with colchicine. In future, there will be hexaploid cotton varieties, having beneficial characteristics of diploid and tetraploid cotton species. According to the special conditions in each of the two applied methods, cotton breeders can use one or both of them. In this study, we show that using of this hormone on the flower for hybrid seeds production is simple; it does not need complex facilities. Therefore, seed production companies will be able to educate experts simply, so that they will do the several crosses during one of the flowering periods of cotton. If produced seeds were weaker in germination, they can use simple culture medium for reforming.

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